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Cytotoxic mechanisms of anti-tumour quinones in parental and resistant lymphoblasts

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Summary The group I aziridinylquinone anti-cancer agents mitomycin C, diaziquone or trenimon were much more cytotoxic to DT-diaphorase-enriched L5178Y/HBM10 lymphoblasts than parental L5178Y cells and caused little oxygen activation. Furthermore, inactivation of cellular DT-diaphorase prevented cytotoxicity whereas catalase did not affect cytotoxicity. This suggests that DT-diaphorase activated these agents and the hydroquinone formed mediated DNA alkylation, crosslinking and cytotoxicity. The group II quinone agents phenanthrenequinone, 2-amino-1,4-naphthoquinoneimine or naphthazarin were also more cytotoxic to L5178Y/HBM10 cells than parental cells and caused considerable oxygen activation. Inactivation of DT-diaphorase, however, prevented both oxygen activation and cytotoxicity. Furthermore added catalase decreased cytotoxicity, whereas catalase inactivation enhanced cytotoxicity. This suggests that DT-diaphorase activated these agents and the hydroquinone formed caused extensive oxygen activation sufficient to cause DNA oxidative damage and cytotoxicity. The group III quinone agents menadione, 2,3-dimethoxy-1,4-naphthoquinone and 2,6-dimethoxy-benzoquinone, on the other hand, were more cytotoxic to the parental cells than L5178Y/HBM10 cells and caused less oxygen activation than group II agents. Furthermore, inactivation of DT-diaphorase enhanced cytotoxicity and prevented oxygen activation than group II agents. Oxygen activation was therefore also attributed to hydroquinone autoxidation. However catalase did not affect cytotoxicity towards parental cells. This suggests that DT-diaphorase detoxified group III quinones and that cytotoxicity may involve DNA oxidative damage by the semiquinone radicals.

Keywords: DT-diaphorase; quinones; cytotoxicity; lymphoblasts

Quinones can undergo either an intracellular two-electron reduction to the hydroquinone or a one-electron reduction to the semiquinone (O'Brien, 1991; Morgan *et al.*, 1992). NAD(P)H:quinone-acceptor oxidoreductase (DT-diaphorase; EC 1.6.99.2) obligatorily catalyses a two-electron quinone reduction. However, the flavoenzymes NADPH-cytochrome P450 reductase, NADH-cytochrome b_5 reductase or NADH-ubiquinone reductase catalyse a one-electron quinone reduction (Miller *et al.*, 1986). The semiquinone or hydroquinone may regenerate the quinone through its reaction with molecular oxygen to produce superoxide radicals $O_2^{\cdot-}$. This futile redox cycle forming toxic oxygen species may culminate in damage to macromolecules and cytotoxicity (Ngo *et al.*, 1991; Nutter *et al.*, 1991). The rate of production of oxygen radicals and the importance of radical reactions in producing cytotoxic damage vary for different quinones and for different environments (Rockwell *et al.*, 1993). The nature and amount of enzymatic reducing systems in cells will also have an influence on the cytotoxic effect of the quinone (Gibson *et al.*, 1992). It is generally agreed that DT-diaphorase plays a role in cells of preventing quinone cytotoxicity, because the hydroquinone formed usually autoxidises less effectively than the semiquinone and can be readily glucuronidated (Lind *et al.*, 1982) and thus eliminated or detoxified.

The L5178Y/HBM10 cell line is probably resistant to the model quinone anti-tumour agent, hydrolysed benzoquinone mustard, because they have a 24-fold increased level of DT-diaphorase relative to the 'wild-type' parental L5178Y cells. The L5178Y/HBM10 cells have also been shown to be 4-fold more sensitive to mitomycin C than parental cells possibly because the hydroquinone metabolite formed by DT diaphorase forms the ultimate DNA alkylating species (Begleiter and Leith, 1990). However, under hypoxic conditions, it is also possible that the semiquinone interacts with DNA (Beall *et al.*, 1994). Recently DT diaphorase gene targeting studies with mouse embryonic stem cells have confirmed that DT diaphorase bioactivates mitomycin C to form cytotoxic metabolites (Yoshida and Tsuda, 1995).

In this study L5178Y/HBM10 cells were also found to be more susceptible than parental cells to phenanthrenequinone, 2-amino-1,4-naphthoquinoneimine and naphthazarin. Evidence is presented suggesting that these quinones are activated by DT diaphorase because their hydroquinones rapidly form cytotoxic active oxygen species. These quinone agents could prove useful for targeting tumour cells containing high DT diaphorase and low catalase. On the other hand menadione, 2,3-dimethoxy-1,4-naphthoquinone and 2,6-dimethoxybenzoquinone were found to be more cytotoxic to parental cells than L5178/HBM10 cells. These quinones seem to be detoxified by DT diaphorase even though their hydroquinones autoxidise. The cytotoxic metabolites formed may therefore be semiquinones rather than reactive oxygen species. These quinone agents could prove useful for targeting tumour cells containing low DT diaphorase.

Materials and methods

Chemicals

Hydrolysed benzoquinone mustard was prepared as described by Begleiter and Leith (1990); 2,3-dimethoxy-1,4-naphthoquinone was prepared as reported by Gant *et al.* (1988) and 2-amino-1,4-naphthoquinoneimine was synthesised as described by Yellow (1975). Horse serum, dicoumarol 3,3'-methylene-bis(4-hydroxycoumarin) and trypan blue were obtained from Sigma (St Louis, MO). Naphthazarin (5,8-dihydroxy-1,4-naphthoquinone) and phenanthrenequinone were obtained from Aldrich Chemical Company (Milwaukee, WI). 3-Amino-1,2,4,-triazole and menadione were purchased from Mann Research Laboratories, NY. Fischer's medium was obtained from Gibco BRL, Burlington, Ontario. Other chemicals were of the highest commercial grade available.

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Materials

Cell culture supplies were obtained from Falcon, Becton Dickinson Labware, (Lincoln Park, NJ), Corning Inc. (Corning, NY), Gelman Sciences Inc. (Ann Arbor, MI). Agar Noble was purchased from Difco Laboratories, Detroit, MI. Bovine liver catalase (50 000 units per mg protein) 2× crystallised was obtained from Sigma. Hydrolysed benzoquinone mustard was prepared as described (Begleiter, 1983).

Cell culture

L5178Y parental and L5178Y/HBM10 resistant mouse lymphoma cells were kindly supplied by Dr A Begleiter (Manitoba Institute of Cell Biology, Winnipeg, Manitoba). Culture conditions for both cell lines were the same as those desribed previously (Silva and O'Brien, 1992). Briefly, cells were grown continuously in Fischer's medium containing 14% horse serum in suspension culture at 37°C in a humidified atmosphere containing 5–7% carbon dioxide. Both cell lines were subcultured every 48 h with suitable dilutions to maintain the cells at a logarithmic growth rate. The L5871Y cell line population density (middle limit of cell number during exponential growth) was maintained at 4×10^4 cells ml^{-1}, whereas the L5178Y/HBM10 cell line population density was maintained at 8×10^4 cells ml^{-1}. The L5178Y/HBM10 cells resistant to hydrolysed benzoquinone mustard [di(2'-hydroxyethyl)amino-1,4-benzoquinone; HBM] were grown continuously in suspension culture in media containing 1 mM HBM. Doubling times were 12 h and 16 h for parental and resistant cells respectively.

Cytotoxicity assay

The parental and/or resistant cells (2×10^5 cells ml^{-1}) were incubated with different concentrations of quinone agents for 1 h at 37°C suspended in media containing horse serum and stirred. The quinone agent was added to the suspension in dimethyl sulphoxide in a 1:200 dilution. The cells were resuspended after 1 h in fresh media. The cytotoxic activity of the quinone agent tested was determined by a soft agar cloning assay as described previously (Begleiter, 1983). Cloning efficiency ranged from 45 to 65% for L5178Y parental cells and from 55 to 80% for L5178Y/HBM10 cells. For inhibition studies, cell suspensions were preincubated for 15 min with 100 μM dicoumarol to inhibit DT-diaphorase or with 200 μM aminotriazole to inhibit catalase. Enzyme assays of the cell homogenates confirmed that DT-diaphorase or catalase had been inactivated 90–95% by these inhibitors (data not shown). Added catalase was also used at a concentration of 4400 units ml^{-1}. Trypan blue (final concentration 0.2%) exclusion was determined to assess cell viability before agar inoculation. However, plasma membrane disruption did not occur at the concentrations used for the clonogenic assay.

Cyanide-resistant respiration

Oxygen uptake was determined using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Model 5300) with a thermostated 2 ml chamber at 37°C. Cellular oxygen consumption was measured at a density of 3×10^6 cells ml^{-1} suspended in Fischer's medium supplemented with 14% horse serum. Potassium cyanide (1 mM) was added to inhibit mitochondrial respiration and 3 μM quinone agent was added after 30 s.

Statistics

Statistical significance of differences between treatments in these studies was determined by Student's *t*-test. The minimal level of significance chosen was $P < 0.05$.

Results

Toxicity towards L5178Y and L5178Y/HBM10 cells

Differences in the cytotoxic susceptibility of the parental (L5178Y) *vs* HBM resistant (L5178Y/HBM10) lymphoma cell lines to quinone-induced toxicity are presented in Table I. The quinone concentration used to achieve less than 1% cell survival varied from 0.5 to 50 μM. The DT-diaphorase-enriched L5178Y/HBM10 cells were more susceptible than parental cells to mitomycin C, diaziquone, trenimon, phenanthrenequinone, 2-amino-1,4-naphthoquinoneimine and naphthazarin suggesting that these quinones were activated by DT-diaphorase. By comparing the concentrations of quinone required to cause the same amount of cytotoxicity it can be concluded that the L5178Y/HBM10 cells were at least 3–4-fold more sensitive to these quinones compared with parental cells. The order of cytostatic effectiveness found was trenimon > mitomycin C > naphthazarin > diaziquone > 2-amino-1,4-naphthoquinoneimine > phenanthrenequinone. On the other hand, the L5178Y/HBM10 cells were at least 4-fold more resistant to menadione, 2,3-dimethoxy-1,4-naphthoquinone, and 2,6-dimethoxy-benzoquinone relative to the parental cells suggesting that the latter quinones were detoxified by DT-diaphorase. The order of cytotoxic effectiveness found for parental cells was 2,3-dimethoxy-1,4-naphthoquinone > 2,6-dimethoxy-benzoquinone > menadione.

Effect of dicoumarol on toxicity

As can be seen in Table I, dicoumarol, a DT-diaphorase inhibitor, markedly decreased the toxicity of mitomycin C, diaziquone, trenimon, phenanthrenequinone, 2-amino-1,4-naphthoquinoneimine and naphthazarin towards the L5178Y/HBM10 cells indicating that DT-diaphorase is responsible for bioactivation in these cells. Enzyme assays of the cell homogenates confirmed that DT-diaphorase had been inactivated by dicoumarol (results not shown). However, dicoumarol had no effect on the toxicity of these quinones towards the parental cell line suggesting that DT-diaphorase was not important in the bioactivation of these agents by these cells. The toxicity of menadione, 2,3-dimethoxy-1,4-naphthoquinone, and 2,6-dimethoxy-benzoquinone towards both cell lines was greatly potentiated by this inhibitor. Dicoumarol by itself at the concentration used did not affect the survival of either cell line.

Table I Toxicity of quinones towards L5178Y and L5178Y/HBM10 cells and the effect of inactivating DT-Diaphorase

		Surviving cell fraction (%)			
	Conc	*L5178Y*		*L5178Y/HBM10*	
Treatment	*(μM)*	*−dic*	*+dic*	*−dic*	*+dic*
Mitomycin C	1.0	0.01	0.01	0.001	0.01
Trenimon	0.01	0.007	0.007	0.001	0.005
Diaziquone	10.0	0.02	0.03	0.001	0.007
Phenanthrene-quinone	50.0	0.076	0.096	0.02	0.15
2-Amino-1,4-naphthoquinone	3.0	0.06	0.05	0.003	0.07
Naphthazarin	0.5	0.04	0.015	0.007	0.05
Menadione	20.0	0.008	0.008	0.035	0.008
2,3-Dimethoxy-1,4-naphthoquinone	1.0	0.00007	0.00007	0.31	0.015
2,6-Dimethoxy-benzoquinone	1.0	0.05	0.05	0.5	0.05

L5178Y and L5178Y/HBM10 cells were incubated with the appropriate quinone agent for 1 h at 37°C in media containing horse serum as described in Materials and methods. Where stated, cells were preincubated with 100μM dicumarol for 15 min to inhibit DT-diaphorase. The cytotoxic activity of the quinone agent tested was determined by a soft agar cloning assay. Values are expressed as means of at least three experiments.

The effect of catalase on toxicity

As shown in Table II the toxicity of phenanthrenequinone, 2-amino-1,4-naphthoquinoneimine and naphthazarin to L5178Y/HBM10 cells was ameliorated by the addition of catalase to the incubation medium of the L5178Y/HBM10 cells. Furthermore, 3-amino-1,2,4,-triazole at a concentration that inhibited intracellular catalase (results not shown) markedly potentiated the toxicity of these three quinones (Table II). Catalase and aminotriazole, however, had no effect on the cytotoxicity induced by menadione, 2,3-dimethoxy-1,4-naphthoquinone, 2,6-dimethoxy-benzoquinone, diaziquone or trenimon in L5178Y cells (Table II) or L5178Y/HBM10 cells (results not shown).

Table II Toxicity of quinones towards L5178Y and L5178Y/HBM10 cells and the effect of catalase on cell survival

Treatment	*Conc (μM)*	*Survivng cell fraction (%)* *Control*	*+catalase*	*+ AT*
L5178Y/HBM10 cells				
Trenimon	0.01	0.0001	0.0001	0.0001
Diaziquinone	10.0	0.02	0.02	0.02
Phenanthrene quinone	50.0	0.020	0.05	0.005
2-Amino-1,4-naphthoquinoneimine	3.0	0.0003	0.001	0.00008
Naphthazarin	0.5	0.005	0.010	0.001
L5178Y cells				
Menadione	20.0	0.008	0.008	0.008
2,3-Dimethoxy-1,4-naphthoquinone	1.0	0.03	0.03	0.05
2,6-Dimethoxy-benzoquinone	1.0	0.05	0.05	0.05

L5178Y and L5178Y/HBM10 cells were incubated with the appropriate quinone agent for 1 h at 37°C in media containing horse serum as described in Materials and methods. Where stated, cells were preincubated with 200μM aminotriazole for 15 min to inhibit catalase. Catalase was used at the concentration of 4400 units ml^{-1} and was added concurrently with the quinone agent. The cytotoxic activity of the quinone agent tested was determined by a soft agar cloning assay. Values are expressed as means of at least three experiments.

Table III Quinone mediated oxygen activation by L5178Y *vs* L5178Y/HBM10 cells

Treatment	*Cyanide-resistant respiration (nmol O_2/3x10^6 cells min^{-1})* *L5178Y* *-dic*	*L5178Y/HBM10* *-dic*	*+dic*
None	1±0.2	1±0.2	1±0.2
Trenimon	2±0.3	1±0.2	2±0.3
Diaziquinone	3±0.3	1±0.2	3±0.3
Phenanthrenequinone	38±4	148±19	53±7
2-Amino-1,4-naphthoquinoneimine	21±2	105±17	32±3
Naphthazarin	5±1	32±4	6±1
Menadione	5±2	8±2	4±1
2,3-Dimethoxy-1,4-naphthoquinone	7±2	19±3	7±2
2,6-Dimethoxy-benzoquinone	5±2	9±2	7±1

Oxygen uptake was determined using a Clark-type oxygen electrode as described in Materials and methods. Cells were suspended in Fischer's medium supplemented by 14% horse serum. Potassium cyanide (1mM) was added before the addition of quinone agent (3μM) to inhibit mitochondrial respiration. Where indicated, dicumarol (100μM) was added before agent addition to inhibit DT-diaphorase. Values are expressed as means of at least three experiments ± s.e.

Cyanide resistant respiration

As shown in Table III, cyanide-resistant respiration induced by group II quinones was greater than that induced by group III quinones, whereas group I quinones were relatively ineffective. Mitomycin C did not induce detectable cyanide-resistant respiration at the concentration used. This oxygen activation by group II and III quinones was 5–6-fold higher in the DT-diaphorase-enriched L5178Y/HBM10 cells than the parental cells. Furthermore, inactivating DT-diaphorase with dicoumarol decreased the rate of cyanide-resistant respiration in the resistant HBM9/10 cells (Table III) but not in the parental cells (data not shown). Group I quinones, however, induced less cyanide-resistant respiration in L5178/HBM10 cells than parental cells.

Discussion

Based on the cytotoxicity results presented in this paper it is possible to divide the investigated quinones into three groups. Group I quinones, consisting of mitomycin C, diaziquone and trenimon as well as Group II quinones, consisting of phenanthrenequinone, 2-amino-1,4-naphthoquinoneimine, and naphthazarin were 3–4-fold more toxic to the HBM-resistant L5178Y/HBM10 cell line than to the L5178Y cell line (Table I). Group III quinones, consisting of menadione, 2,3-dimethoxy-1,4-naphthoquinone, and 2,6-dimethoxy-benzoquinone were, however, much more toxic to the L5178Y cell line than the L5178Y/HBM10 cell line.

The DT-diaphorase activity of the L5178Y/HBM10 cell line was 24-fold higher than that of the parental cell line (Begleiter *et al.*, 1988). The above cytotoxicity results could therefore be explained if DT-diaphorase reductively bioactivates group I and II quinones to their toxic species but reductively detoxifies group III quinones. Evidence in support of this was that when cellular DT-diaphorase was inhibited by the specific inhibitor, dicoumarol (Preusch *et al.*, 1991), the toxicity of group I and II quinones towards the L5178Y/HBM10 cells was prevented, whereas the toxicity of group III quinones was greatly potentiated. Tsuda (1990) also found a potentiation of menadione toxicity in cultured mammalian fibroblast cells when DT-diaphorase was inactivated. They hypothesised that DT-diaphorase prevented the oxidative stress that was responsible for cytotoxicity. The lack of effect of dicoumarol on the quinone susceptibility of parental L5178Y cells suggests that their DT-diaphorase activity is too low to compete with other quinone reductases.

The molecular cytotoxic mechanism for the aziridinylquinones mitomycin C, diaziquone and trenimon could not be attributed to oxygen activation as inactivation of intracellular catalase or addition of catalase did not affect cytotoxicity. Furthermore, cyanide-resistant respiration induced by group I quinones was much less than that found for group II and III quinones. In any case, as shown previously, using higher trenimon concentrations, cyanide-resistant respiration was much lower in L5178Y/HBM10 cells than parental cells but became similar to parental cells when DT-diaphorase as inhibited with dicoumarol (Silva and O'Brien, 1992). This suggests that the semiquinone radical of group I quinone and not hydroquinone is responsible for oxygen activation (Silva and O'Brien, 1992). Begleiter *et al.* (1989) also previously found that L5178Y/HBM10 cells were 4-fold more susceptible to mitomycin C than parental cells and had a 5-fold greater level of DNA–DNA crosslinks. Although GSH levels and catalase activity were higher in L5178Y/HBM10 cells, increased DT-diaphorase levels were possibly responsible for the increased cell susceptibility as inactivation of DT-diaphorase prevented cytotoxicity and DNA crosslinking. This suggests that the hydroquinone metabolite of trenimon forms an electrophilic quinone methide that alkylates DNA. Similar conclusions were reached for diaziquone (Gibson *et al.*, 1992). Siegel *et al.* (1992) also showed that purified DT-diaphorase catalysed mitomycin C-induced DNA crosslinking

particularly at lower pH values, but resulted in the alkylation and inactivation of DT-diaphorase (Siegel *et al.*, 1993). Furthermore, high DT-diaphorase activity correlated with aerobic mitomycin C sensitivity in human fibroblasts derived from members of a cancer-prone family (Marshall *et al.*, 1991) and human non-small-cell lung carcinoma (Malkinson *et al.*, 1992). A mitomycin C-resistant human colon cancer cell line also had very low DT-diaphorase activity (Pan *et al.*, 1993).

Evidence that the molecular cytotoxic mechanism for group II quinones involves oxygen activation is that the inhibition of endogenous catalase by aminotriazole significantly increased the susceptibility of these cells to group II quinones but did not affect cell susceptibility to group III quinones. Cyanide-resistant respiration with these quinones was also much higher than that found with group I or III quinones. Similar results were found with isolated hepatocytes (O'Brien, 1991). It was also much higher in L5178Y/HBM10 cells than parental cells and furthermore, added catalase also prevented cytotoxicity of group II quinones but not group III quinones.

Furthermore, the ferric ion chelator desferoxamine markedly decreased group II quinone but not group I or III quinone cytotoxicity (results not shown). This suggests that cytotoxicity is mediated by reactive oxygen species as ferric iron is required for the formation of reactive oxygen species by the Haber–Weiss reaction.

Group II quinones therefore likely activate oxygen by a two electron futile redox cycle involving the formation of hydroquinones which autoxidise and generate cytotoxic reactive oxygen species. The hydroquinones of phenanthrenequinone, 2,3-dimethoxy-1,4-naphthoquinone and naphthazarin rapidly autoxidise probably because of their lower redox potential and lower pK_a. The autoxidation species involved is the dianionic species (Q^{2-}), (O'Brien, 1991; Ollinger *et al.*, 1990). Naphthazarin has a low pK_a pf 7.6, because of the stability of the hydrogen-bonded structure (Land *et al.*, 1982). The semiquinone radicals formed when 2-amino-1,4-naphthoquinoneimine was added to CHO cells can also activate oxygen (Powis *et al.*, 1987).

Inactivation of DT-diaphorase increased the susceptibility of L5178/HBM10 cells to group III quinones. Others have also shown that the susceptibility of cultured mammalian fibroblastic cells to menadione is increased by inhibiting DT-diaphorase (Tsuda, 1990). However, the lack of effect of exogenous catalase or inactivation of intracellular catalase on group III quinone toxicity in contrast to group II quinone cytotoxicity suggests that the cytotoxic mechanism of these two groups are different. Group III quinones also underwent less oxygen activation by futile redox cycling than group II quinones in HBM-resistant cells (Table III). Oxygen activation by group III quinones was, however, still greater in L5178/HBM10 cells than parental cells and was prevented by inactivation of DT-diaphorase. This suggests that oxygen activation can be attributed to hydroquinone auto-oxidation but is not sufficient to cause cytotoxicity. The greater cytotoxicity of these quinones towards parental cells could be attributed to semiquinone radicals interacting with DNA. Semiquinone or quinones have been shown to bind covalently to DNA (Dwivedy *et al.*, 1992). Previously Pething *et al.* (1984) found a direct correlation between the electrochemical potentials, generated semiquinone lifetimes, and cytotoxic action in Ehrlich ascites-bearing mice of dimethoxy-p-benzoquinones.

Cantoni *et al.* (1991), using the intracellular ion chelator 1, 10-phenanthroline, showed that the hydrogen peroxide produced during the metabolism of menadione is only partly responsible for the induction of DNA breaks and that this activated oxygen type of lesion did not result in cytotoxicity.

An increased cellular expression of DT-diaphorase in transformed cells often occurs during chemical carcinogenesis and further increases are usually associated with resistance to chemotherapy. These tumours should therefore be particularly susceptible to Group I (aziridinylquinone anti-cancer agents) or Group II quinones under aerobic conditions. Measurement of DT-diaphorase in tumour biopsies known as 'enzyme profiling' would assist in the selection of patients most likely to benefit from these DT-diaphorase-directed agents (Riley and Workman, 1992). Indeed, certain human colon tumours and non-small-cell lung tumours which are difficult to treat have high DT-diaphorase activity relative to surrounding normal tissue (Malkinson *et al.*, 1992).

In conclusion these results could be explained as follows: the group I aziridinylquinones are activated by DT-diaphorase since their respective hydroquinones readily alkylate and crosslink DNA bases. Group II hydroquinones intercalate DNA bases and auto-oxidise to form reactive oxygen species which damage DNA. Group III quinones are, however, detoxified by DT-diaphorase probably because their hydroquinones auto-oxidise less readily than that of group II and/or do not intercalate DNA. Parental cells are, however, susceptible to Group III semiquinone radicals by a mechanism that does not involve oxygen activation. Group II quinone agents may therefore prove useful for targeting tumour cells containing high DT-diaphorase activity and low catalase activity. On the other hand, Group III quinone agents could prove useful for targeting tumour cells containing low DT-dipahorase activity.

Abbreviations

HBM, hydrolysed benzoquinone mustard, [di(2'-hydroxyethyl)amino-1,4-benzoquinone]; dic, dicoumarol; AT, 3-amino-1,2,4-triazole; KCN, potassium cyanide.

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